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(54) Title: PATHOGENIC *ESCHERICHIA COLI* ASSOCIATED PROTEIN

(57) Abstract

The present invention provides the EspA polypeptide, which is secreted by pathogenic *E. coli*, such as the enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli*. Diagnosis of disease caused by such pathogenic *E. coli* can be performed by standard techniques, such as those based upon the use of antibodies which bind to EspA to detect the protein, as well as those based on the use of nucleic acid probes for detection of nucleic acids encoding EspA protein. The invention also provides isolated nucleic acid sequences encoding EspA, EspA polypeptide, EspA peptides, a method for producing recombinant EspA, antibodies which bind to EspA, and a kit for the detection of EspA-producing *E. coli*. The invention also provides a method of immunizing a host with EspA to induce a protective immune response to EspA.

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PATHOGENIC *ESCHERICHIA COLI* ASSOCIATED PROTEIN

Cross Reference to Related Application

This application claims priority from U.S. Provisional Application 60/015,999, filed April 23, 1996.

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This invention was made with support from Public Health Service award AI32074 from the National Institutes of Health. The United States government may have certain rights in this invention.

Field of the Invention

The present invention relates generally to the virulence of pathogenic organisms and more specifically to virulence factors associated with enteropathogenic bacteria.

Background of the Invention

Antibiotics have been used for years to successfully treat diverse bacterial infections. However, bacterial resistance to antibiotics has been an increasing problem over the past few years. Many pathogens are now resistant to several antibiotics, and in some cases, the diseases they cause are no longer treatable with conventional antibiotics. Despite the past successes of antibiotics, there have been few, if any, new classes of antibiotics developed in the past two decades. New variations on existing drugs have been introduced, but resistance to these compounds usually arises within a short period of time.

Many studies have shown that if a mutation is made in a gene that encodes a virulence factor, the organism containing that gene is no longer pathogenic.

Additionally, if a host is vaccinated against a virulence factor, disease can often be blocked. However, it has not been shown that specific inhibition of a virulence factor can attenuate disease.

The mechanisms of action for toxins, adherence, invasion, intracellular parasitism, have been studied. However, each virulence factor uses a different mechanism, which has made the development of a broad spectrum inhibitor impossible. One conserved factor that could be considered a target for a therapeutic is a two-component regulatory system. However, this system is not specific for virulence factors, and is used in several bacterial housekeeping systems. Additionally, the systems have been identified in eukaryotic systems, which would increase the risk of host toxicity if an inhibitor was utilized. To develop an ideal anti-infective agent, the bacterial virulence mechanism that the antibiotic affects should be universal for many pathogens, specific for virulence mechanisms, and not be present in host cells. One such system that has recently been identified is the bacterial type III secretion system.

Gram-negative bacteria utilize specialized machinery to export molecules across their two membranes and the piroplasm, a process critical for moving virulence factors to the bacterial surface where they can interact with host components. Gram-negative secretion has been divided into four major pathways. First, the Type I secretion is used by a small family of toxins, with *E. coli* hemolysin being the prototype. Second, the type II secretion system is the major export pathway used by most Gram-negative bacteria to export many molecules, including some virulence factors; it shares homology to mammalian drug resistance mechanisms. Third, the type IV secretion system is encoded within the secreted product, which cleaves itself as part of the secretion mechanism; the prototype of this system is the *Neisseria* IgA protease. Fourth, the most recently discovered secretion pathway, is the type III pathway.

Type III secretion systems were originally described as a secretion system for *Yersinia* secreted virulence proteins, YOPs, which are critical for *Yersinia* virulence. A homologous secretion system was then identified in several plant pathogens, including

Pseudomonas syringae, P. solanacearum, and *Xantharnonas carnpestris*. These plant pathogens use this secretion pathway to secrete virulence factors (harpins and others) that are required for causing disease in plants. Although the secretion system is similar, harpins and YOPs (*i.e.* the secreted virulence factors) are not homologous polypeptides. Several other type III secretion systems necessary for virulence have more recently been identified in other pathogens. These systems include the invasion systems *Salmonella* and *Shigella* use to enter cells and cause disease. Another type III secretion system has been identified in *Salmonella* which is critical for disease, although the secreted products of this pathway and the virulence mechanisms have not been established yet. *Pseudomonas aeruginosa* has a type III secretion system necessary for secretion of Exoenzyme S, a potent virulence factor.

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infant diarrhea and was the first *E. coli* shown to cause gastroenteritis. Enteropathogenic *E. coli* activates the host epithelial cells' signal transduction pathways and causes cytoskeletal rearrangement, along with pedestal and attaching/effacing lesion formation.

A three-stage model has describes enteropathogenic *E. coli* pathogenesis. An initial localized adherence to epithelial cells, mediated by a type IV fimbria, is followed by the activation of host epithelial cell signal transduction pathways and intimate attachment to host epithelial cells. These final two steps are collectively known as attaching and effacing. The signal transduction in the host epithelial cells involves activation of host cell tyrosine kinase activity leading to tyrosine phosphorylation of a 90 kilodalton host membrane protein, Hp90, and fluxes of intracellular inositol phosphate (IP_3) and calcium. Following this signal transduction, the bacteria adheres intimately to the surface of the epithelial cell, accompanied by damage to host epithelial cell microvilli and accumulation of cytoskeletal proteins beneath the bacteria.

Summary of the Invention

The present invention is based on the discovery a protein associated with virulence in pathogenic bacteria, for example enteropathogenic *E. coli*.

DNA sequence analysis of the Locus of Enterocyte Effacement between *eae*A and *esp*B identified a gene (*esp*A) that matched the amino terminal sequence of the 25 kilodalton enteropathogenic *E. coli* secreted protein. A mutant with an insertion in *esp*A does not secrete this protein, activate epithelial cell signal transduction or cause cytoskeletal rearrangement. However, these functions could be complemented by a cloned wild-type *esp*A gene.

Two enteropathogenic *E. coli* genes, *esp*A and *esp*B, that encode secreted virulence factors, EspA and EspB respectively, were cloned and sequenced. These proteins were shown to be involved in the triggering of host epithelial signal transduction pathways and invasion. Since EspA is a secreted protein, it is ideally suited for use in a fusion protein linked to a polypeptide of interest.

The type III secretion pathway is an ideal target for potential inhibitors, because it is a virulence factor-specific conserved pathway identified in bacteria. The present invention provides a method for identifying inhibitors of type III secretion systems. The relevance of this invention is directed toward the development of new antibacterial therapeutics. In contrast to other antibiotics, the compounds identified by the method of this invention will not kill or inhibit growth of pathogens; instead, the compounds will block the secretion of virulence factors that are critical to causing disease. Because the type III secretion system is the first virulence mechanism that shows a large degree of conservation between diverse pathogens, some of the compounds identified by the method of this invention will be broad spectrum therapeutics. The benefit would be the identification of new therapeutics that could be used in the treatment of several human, animal, and plant diseases.

Brief Description of the Drawings

FIGURE 1 shows the nucleotide and deduced amino acid sequence of *espA* (herein referred to as SEQ ID NO: 1 and SEQ ID NO:2, respectively). Potential ribosome binding sites are underlined. Nucleotides included in primers Donne-99 and Donne-100, flanking the deletion engineered in pLCL121, are shaded.

FIGURE 2 shows the construction of a non-polar mutation in *espA*. Primers Donne-90 and the reverse primer were used to amplify a fragment containing a 5' portion of the gene, which was cloned into pCRscript to create pLCL119. Primers Donne-100 and the universal primer were used to amplify a fragment containing a 3' portion of the gene, which was cloned into pCRscript to create pLCL120. New NruI sites were incorporated into both Donne-99 and Donne-100 so that the NruI-SalI fragment of pLCL120 could be cloned into pLCL119 to create pLCL121, which has a 150bp deletion within the *espA* gene. A *SmaI* fragment from pUC18K containing the *aphA*-3 kanamycin resistance gene was cloned into the *NruI* site of pLCL121 to create pLCL122. This insertion results in a transcriptional fusion of the *aphA*-3 gene and a translational fusion of the 3' end of the *espA* gene, with preservation of the *espA* reading frame. The ribosome binding sites are underlined.

FIGURE 3 shows a genetic map of the plasmids containing RDEC-1 (A) and enteropathogenic *Escherichia coli* (EPEC) (B) *esp*A, *esp*D, and *esp*B genes. Arrows indicate positions that stop codon insertions were made in *esp*A and *esp*B (A), and the frame shift mutation engineered into the BglII site in espD (B). The 250 base pair deletion in *esp*B is marked by / /. Solid and clear boxes represent open reading frames and predicted open reading frames. Restriction enzymes are indicated as follows: Bam, BamHI; Ec, EcoRI; Bg, BglII; Xb, XbaI; Sa, SalI.

FIGURE 4 shows the nucleotide sequence of RDEC-1 *esp*A (A) (herein referred to as SEQ ID NO: 3 and SEQ ID NO:4, respectively) and *esp*B (B) (herein referred to as SEQ ID NO: 5 and SEQ ID NO:6, respectively). Asterisks indicate stop codons. Potential ribosome binding sites are underlined. Predicted amino acid sequences of EspA and EspB are aligned in C) (SEQ ID NO:7-14). Shaded areas represent identity. Nucleotide and amino acid sequences have been deposited into the EMBL GenBank and their accession numbers are as follows: RDEC-1 *esp*A (U80908), RDEC-1 *esp*B (U80796), enteropathogenic *Escherichia coli* strain E2348/69 *esp*A (Z54352), enteropathogenic *Escherichia coli* strain E2348/69 *esp*B (Z21555), enteropathogenic *Escherichia coli* strain E2348/69 espD (Y09228), enterohemorrhagic *Escherichia coli* strain EDL933 serotype 0157 *esp*B (X96953), enterohemorrhagic *Escherichia coli* strain 413/89-1 serotype 026 *esp*B (X99670).

Detailed Description

The present invention provides a polypeptide, called EspA, which is secreted by pathogenic *E. coli*, such as the enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli*. Diagnosis of disease caused by such pathogenic *E. coli* can be performed by standard techniques, such as those based upon the use of antibodies which bind to EspA to detect the protein, as well as those based on the use of nucleic acid probes for detection of nucleic acids encoding EspA polypeptide. The invention also provides isolated nucleic acid sequences encoding EspA polypeptide, EspA peptides, a recombinant method for producing recombinant EspA, antibodies which bind to EspA, and a kit for the detection of EspA-producing *E. coli*. The invention also provides a method of immunizing a host with EspA to induce a protective immune response to EspA.

The details of the preferred embodiments of the present invention are set forth in the accompanying drawings and the description below. Once the details of the

As used herein, the term "EspA" (for EPEC secreted [or signaling] protein A) refers to a polypeptide which is a secreted protein from enteropathogenic or enterohemorrhagic *E. coli* and has a molecular weight of about 25 kilodaltons as determined by SDS-PAGE. EspA is an enteropathogenic *E. coli*-secreted protein necessary for activating epithelial cell signal transduction, intimate contact, and formation of attaching and effacing lesions, processes correlated with disease. An example of epithelial cells are cells.

As used herein, the term "polypeptide" encompasses any naturally occurring allelic variant thereof as well as manufactured recombinant forms. As used herein, EspA polypeptides encompass both naturally occurring and recombinant forms, *i.e.*, non-naturally occurring forms of the protein and the peptide that are sufficiently identical to naturally occurring EspA peptide to have a similar function of causing pathogenicity. Examples of such polypeptides include the EspA polypeptides from enteropathogenic and enterohemorrhagic *E. coli,* but are not limited to them. Protein and polypeptides include derivatives, analogs and peptidomimetics. Alternatively, EspA peptides can be chemically synthesized using synthesis procedures known to one skilled in the art. Preferably, an automated peptide synthesizer is used with N$^{\alpha}$Fmoc amino acids on a polyethylene glycol-polystyrene (PEGPS) graft resin. Suitable linkers such as a peptide amide linker (PAL) can be used, for example, to create carboxamide end groups.

The term "substantially pure" is used herein to describe a molecule, such as a polypeptide (*e.g.*, an EspA polypeptide, or a fragment thereof) that is substantially free of other proteins, lipids, carbohydrates, nucleic acids, and other biological materials with which it is naturally associated. For example, a substantially pure molecule, such as a polypeptide, can be at least 60%, by dry weight, the molecule of interest. One skilled in the art can purify EspA polypeptides using standard protein purification methods and the purity of the polypeptides can be determined using standard methods including, *e.g.*, polyacrylamide gel electrophoresis (*e.g.*, SDS-PAGE), column chromatography (*e.g.*,

high performance liquid chromatography (HPLC)), and amino-terminal amino acid sequence analysis.

EspA polypeptides included in the invention can have one of the amino acid sequences of EspAs from human or rabbit enteropathogenic *E. coli*, for example, the amino acid sequence of Figure 1 or Figure 4. EspA polypeptides, such as those shown in Figures 1 and 4, can be characterized by being approximately 25 kD as determined by SDS-PAGE.

Also included in the invention are polypeptides having sequences that are "substantially identical" to the sequence of an EspA polypeptide, such as one of EspAs in Figures 1 and 4. A "substantially identical" amino acid sequence is a sequence that differs from a reference sequence only by conservative amino acid substitutions, for example, substitutions of one amino acid for another of the same class (*e.g.*, substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine), or by one or more non-conservative substitutions, deletions, or insertions, provided that the polypeptide retains at least one EspA-specific activity or an EspA-specific epitope. For example, one or more amino acids can be deleted from an EspA polypeptide, resulting in modification of the structure of the polypeptide, without significantly altering its biological activity. For example, amino- or carboxyl-terminal amino acids that are not required for EspA biological activity, can be removed. Such modifications can result in the development of smaller active EspA polypeptides.

Other EspA polypeptides included in the invention are polypeptides having amino acid sequences that are at least 50% identical to the amino acid sequence of an EspA polypeptide, such as any of EspAs in Figures 1 and 4. The length of comparison in determining amino acid sequence homology can be, for example, at least 15 amino acids, for example, at least 20, 25, or 35 amino acids. Homology can be measured using standard sequence analysis software (*e.g.*, Sequence Analysis Software Package of the

Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705; also see Ausubel, *et al.*, *supra*).

The invention also includes fragments of EspA polypeptides that retain at least one EspA-specific activity or epitope. For example, an EspA polypeptide fragment containing, *e.g.*, at least 8-10 amino acids can be used as an immunogen in the production of EspA-specific antibodies. The fragment can contain, for example, an amino acid sequence that is conserved in EspAs. In addition to their use as peptide immunogens, the above-described EspA fragments can be used in immunoassays, such as ELISAs, to detect the presence of EspA-specific antibodies in samples.

The EspA polypeptides of the invention can be obtained using any of several standard methods. For example, EspA polypeptides can be produced in a standard recombinant expression systems (see below), chemically synthesized (this approach may be limited to small EspA peptide fragments), or purified from tissues in which they are naturally expressed (see, *e.g.*, Ausubel, *et al.*, *supra*).

The invention also provides isolated nucleic acid molecules that encode the EspA polypeptides described above, as well as fragments thereof. For example, nucleic acids that encode EspAs as in Figures 1 and 4 are included in the invention. These nucleic acids can contain naturally occurring nucleotide sequences (see Figures 1 and 4), or sequences that differ from those of the naturally occurring nucleic acids that encode EspAs, but encode the same amino acids, due to the degeneracy of the genetic code. The nucleic acids of the invention can contain DNA or RNA nucleotides, or combinations or modifications thereof.

By "isolated nucleic acid" is meant a nucleic acid, *e.g.*, a DNA or RNA molecule, that is not immediately contiguous with the 5' and 3' flanking sequences with which it normally is immediately contiguous when present in the naturally occurring genome of the organism from which it is derived. The term thus describes, for example, a nucleic acid that is incorporated into a vector, such as a plasmid or viral vector; a nucleic acid that is incorporated into the genome of a heterologous cell (or the genome of

a homologous cell, but at a site different from that at which it naturally occurs); and a nucleic acid that exists as a separate molecule, *e.g.*, a DNA fragment produced by PCR amplification or restriction enzyme digestion, or an RNA molecule produced by *in vitro* transcription. The term also describes a recombinant nucleic acid that forms part of a hybrid gene encoding additional polypeptide sequences that can be used, for example, in the production of a fusion protein.

The nucleic acid molecules of the invention can be used as templates in standard methods for production of EspA gene products (*e.g.*, EspA RNAs and EspA polypeptides; see below). In addition, the nucleic acid molecules that encode EspA polypeptides (and fragments thereof) and related nucleic acids, such as (1) nucleic acids containing sequences that are complementary to, or that hybridize to, nucleic acids encoding EspA polypeptides, or fragments thereof (*e.g.*, fragments containing at least 12, 15, 20, or 25 nucleotides); and (2) nucleic acids containing sequences that hybridize to sequences that are complementary to nucleic acids encoding EspA polypeptides, or fragments thereof (*e.g.*, fragments containing at least 12, 15, 20, or 25 nucleotides); can be used in methods focused on their hybridization properties. For example, as is described in further detail below, such nucleic acid molecules can be used in the following methods: PCR methods for synthesizing EspA nucleic acids, methods for detecting the presence of an EspA nucleic acid in a sample, screening methods for identifying nucleic acids encoding new EspA family members, and therapeutic methods.

The invention also includes methods for identifying nucleic acid molecules that encode members of the EspA polypeptide family in addition to EspAs shown in Figures 1 and 4. In these methods, a sample, *e.g.*, a nucleic acid library, such as a cDNA library, that contains a nucleic acid encoding an EspA polypeptide is screened with an EspA-specific probe, *e.g.*, an EspA-sp. cific nucleic acid probe. EspA-specific nucleic acid probes are nucleic acid molecules (*e.g.*, molecules containing DNA or RNA nucleotides, or combinations or modifications thereof) that specifically hybridize to nucleic acids encoding EspA polypeptides, or to complementary sequences thereof. The

term "EspA-specific probe," in the context of this method of invention, refers to probes that bind to nucleic acids encoding EspA polypeptides, or to complementary sequences thereof, to a detectably greater extent than to nucleic acids encoding other polypeptides, or to complementary sequences thereof. The term "EspA-specific probe" thus includes probes that can bind to nucleic acids encoding EspA polypeptides (or to complementary sequences thereof).

The invention facilitates production of EspA-specific nucleic acid probes. Methods for obtaining such probes can be designed based on the amino acid sequence alignments shown in Figures 1-3. The probes, which can contain at least 12, *e.g.*, at least 15, 25, 35, 50, 100, or 150 nucleotides, can be produced using any of several standard methods (see, *e.g.*, Ausubel, *et al., supra*). For example, preferably, the probes are generated using PCR amplification methods . In these methods, primers are designed that correspond to EspA-conserved sequences, which can include EspA-specific amino acids, and the resulting PCR product is used as a probe to screen a nucleic acid library, such as a cDNA library. A nucleotide sequence encoding EspA was identified generally following this process based upon the analysis of the sequences of EspA in Figures 1 and 4.

As is known in the art, PCR primers are typically designed to contain at least 15 nucleotides, for example 15-30 nucleotides. The design of EspA-specific primers containing 21 nucleotides, which encode EspA peptides containing 7 amino acids, are described as follows. Preferably, most or all of the nucleotides in such a probe encode EspA-conserved amino acids, including EspA-specific amino acids. For example, primers containing sequences encoding peptides containing at least 40% EspA-conserved amino acids can be used. Such a primer, containing 21 nucleotides, can include sequences encoding at least 3 EspA-conserved amino acids. Thus, the primer can contain sequences encoding at least one EspA-specific amino acid, for example, up to 7 EspA-specific amino acids. Once EspA-specific amino acid sequences are selected as templates against which primer sequences are to be designed, the primers can be

synthesized using, *e.g.*, standard chemical methods. As is described above, due to the degeneracy of the genetic code, such primers should be designed to include appropriate degenerate sequences, as can readily be determined by one skilled in the art.

As used herein, the term "*esp*A" refers to polynucleotide encoding the EspA polypeptide. These polynucleotides include DNA, cDNA and RNA sequences which encode EspA. All polynucleotides encoding all or a portion of EspA are also included herein. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, a *esp*A polynucleotide can be subjected to site-directed mutagenesis. The *esp*A polynucleotide sequence also includes antisense sequences. All degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of EspA peptide encoded by the nucleotide sequence is functionally unchanged.

This invention encompasses nucleic acid molecules that hybridize to the polynucleotide of the invention. As used herein, the term "nucleic acid" encompasses RNA as well as single and double-stranded DNA and cDNA. The polynucleotide encoding EspA includes the nucleotide sequence in FIGURE 1 and 4, as well as nucleic acid sequences complementary to that sequence. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxynucleotides A, G, C, and T of FIGURE 1 and 4 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the protein of FIGURE 1 or 4 under physiological conditions.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting

hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization techniques which are well known in the art. These include, but are not limited to (1) hybridization of libraries with probes to detect homologous nucleotide sequences, (2) polymerase chain reaction (PCR) on DNA using primers capable of annealing to the DNA sequence of interest, and (3) antibody screening of expression libraries to detect cloned DNA fragments with shared structural features.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically or produced by fragmentation of the native sequence. Chemical synthesis requires that short, oligopeptide stretches of amino acid sequence be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either

single-stranded DNA or denatured double-stranded DNA. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned.

The invention provides nucleic acid sequences encoding the EspA polypeptides, vectors and host cells containing them and methods of expression. After a peptide of EspA is isolated, nucleic acids encoding the peptide can be isolated by methods well known in the art. These isolated nucleic acids can be ligated into vectors and introduced into suitable host cells for expression. Methods of ligation and expression of nucleic acids within cells are well known in the art (*see*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989, incorporated herein by reference).

As used herein, the terms "*esp*B" and "*eae*A" refer to genes other than *esp*A that encode enteropathogenic *E. coli*-secreted proteins. As used herein, the term "EspB" and "EaeA" refer to the proteins encoded by the *esp*B and the *eae*A genes, respectively.

The invention provides vectors containing polynucleotides encoding the EspA polypeptide. For example, the plasmid (pMSD2) with an intact *esp*A can restore secretion of the EspA protein in an *esp*A deficient strain. As used herein, "vectors" includes plasmids, DNA and RNA viral vectors, baculoviral vectors, vectors for use in yeast, and other vectors well known to those of skill in the art. Several types of vectors are commercially available and can be used to practice this invention. Examples of vectors useful in the practice of this invention include those as widely varied as the low-copy vector pMW118, the positive-selection suicide vector pCVD442, and the commercially available pBluescript II SK(+) (Stragene, La Jolla, CA).

When the vector is a plasmid, it generally contains a variety of components including promoters, signal sequences, phenotypic selection genes, origins of replication sites. nd other necessary components as are known to those of skill in the art. Promoters most commonly used in prokaryotic vectors include the *lacZ* promoter system, the alkaline phosphatase pho A promoter, the bacteriophage λPL promoter (a temperature sensitive promotor), the *tac* promoter (a hybrid *trp-lac* promoter regulated by the *lac*

repressor), the tryptophan promoter, and the bacteriophage T7 promoter. For example, the low-copy vector pMW118 under control of the *lacZ* promoter.

A signal sequence is typically found immediately 5' to the nucleic acid encoding the peptide, and will thus be transcribed at the amino terminus of the fusion protein.

Typical phenotypic selection genes are those encoding proteins that confer antibiotic resistance upon the host cell. For example, ampicillin resistance gene (*amp*) and the tetracycline resistance gene (*tet*) are readily employed for this purpose. For a different example, the *aph*A-3 cassette, encoding a gene for resistance to kanamycin (*kan*), may be cloned into the region of vector containing polynucleotides encoding the EspA polypeptide for selection of the vector on kanamycin plates.

Construction of suitable vectors containing polynucleotides encoding EspA polypeptide are prepared using standard recombinant DNA procedures well known to those of skill in the art. Isolated polynucleotides encoding the EspA polypeptide to be combined to form the vector are cleaved and ligated together in a specific order and orientation to generate the desired vector.

The invention provides a host cell containing a vector having a polynucleotide encoding the EspA polypeptide. The polynucleotides of the present invention can be used to produce transformed or transfected cells for enhanced production of the expressed EspA. EspA can be isolated from transformed cells by standard methods well known to those of skill in the art. The protein could be isolated, for example, using immunoaffinity purification.

DNA sequences encoding EspA can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included

when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the EspA polynucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the EspA genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells.

Polynucleotide sequences encoding EspA can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell if desired. For another example, triparental conjugation may be used to genetically introduce vector into *E. coli*, especially enteropathogenic *E. coli* or rabbit enteropathogenic *E. coli*. The transformed cells are selected by growth on an antibiotic, commonly tetracycline (*tet*) or ampicillin (*amp*), to which they are rendered resistant due to the presence of *tet* or *amp* resistance genes on the vector. In a specific embodiment, cells are selected on the basis of resistance to kanamycin and sucrose.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as micro-injection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the EspA of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (see for example, *Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Isolation and purification of microbial expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

Among the prokaryotic organisms which may serve as host cells are *E. coli* strain JM101, *E. coli* K12 strain 294 (ATCC number 31,446), *E. coli* strain W3110 (ATCC number 27,325), *E. coli* X1776 (ATCC number 31, 537), *E. coli* XL-1Blue (Stratagene), and *E. coli* B; however, many other strains of *E. coli*, such as HB101, NM522, NM538, NM539 and many other species and genera of prokaryotes can be used as well. Besides the *E. coli* strains listed above, bacilli such as *Bacillus subtillis*, other enterobacteriaceae such as *Salmonella typhimunium* or *Serratia marcesans* and various *Pseudomonas* species can all be used as hosts. In one specific embodiment, the prokaryotic host cell is enteropathogenic *E. coli*. In another specific embodiment, the prokaryotic host cell is rabbit enteropathogenic *E. coli*.

Among the eukaryotic organisms which may serve as host cells are yeast strains such as PS23-6A, W301-18A, LL20, D234-3, INVSC1, INVSC2, YJJ337. Promoter and enhancer sequences such as gal 1 and pEFT-1 are useful. Vra-4 also

provides a suitable enhancer sequence. Sequences useful as functional origins of replication include ars1 and 2μ circular plasmid.

The Gram-negative bacteria are a diverse group of organisms and include Spirochaetes such as *Treponema* and *Borrelia*, Gram-negative bacilli including the Pseudomonadaceae, Legionellaceae, Enterobacteriaceae, Vibrionaceae, Pasteurellaceae, Gram-negative cocci such as Neisseriaceae, anaerobic Bacteroides, and other Gram-negative bacteria including *Rickettsia, Chlamydia*, and Mycoplasma.

Gram-negative bacilli (rods) are important in clinical medicine. They include (1) the Enterobacteriaceae, a family which comprises many important pathogenic genera, (2) *Vibrio, Campylobacter* and *Helicobacter* genera, (3) opportunistic organisms (*e.g., Pseudomonas, Flavobacterium,* and others) and (4) *Haemophilus* and *Bordetella* genera. The Gram-negative bacilli are the principal organisms found in infections of the abdominal viscera, peritoneum, and urinary tract, as well secondary invaders of the respiratory tracts, burned or traumatized skin, and sites of decreased host resistance. Currently, they are the most frequent cause of life-threatening bacteremia. Examples of pathogenic Gram-negative bacilli are *E. coli* (diarrhea, urinary tract infection, meningitis in the newborn), *Shigella* species (dysentery), *Salmonella typhi* (typhoid fever), *Salmonella typhimurium* (gastroenteritis), *Yersinia enterocolitica* (enterocolitis), *Yersinia pestis* (black plague), *Vibrio cholerae* (cholera), *Campylobacter jejuni* (enterocolitis), *Helicobacter jejuni* (gastritis, peptic ulcer), *Pseudomonas aeruginosa* (opportunistic infections including burns, urinary tract, respiratory tract, wound infections, and primary infections of the skin, eye and ear), *Haemophilus influenzae* (meningitis in children, epiglottitis, otitis media, sinusitis, and bronchitis), and *Bordetella pertussis* (whooping cough). *Vibrio* is a genus of motile, Gram-negative rod-shaped bacteria (family Vibrionaceae). *Vibrio cholerae* causes cholera in humans; other species of *Vibrio* cause animal diseases. *E. coli* colonize the intestines of humans and warm blooded animals, where they are part of the commensal flora, but there are types of *E. coli* that cause human and animal intestinal diseases. They include the enteroaggregative *E. coli*

(EaggEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E.coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC). Uropathogenic *E. coli* (UPEC) cause urinary tract infections. There are also neonatal meningitis *E. coli* (NMEC). Apart from causing similar infections in animals as some of the human ones, there are specific animal diseases including: calf septicaemia, bovine mastitis, porcine oedema disease, and air sac disease in poultry.

The *Neisseria* species include *N. cinerea, N. gonorrhoeae, N. gonorrhoeae* subsp. *kochii, N. lactamica, N. meningitidis, N. polysaccharea, N. mucosa, N. sicca, N. subflava*, the asaccharolytic species *N. flavescens, N. caviae, N. cuniculi* and *N. ovis*. The strains of *Moraxella (Branhamella) catarrhalis* are also considered by some taxonomists to be *Neisseria*. Other related species include *Kingella, Eikenella, Simonsiella, Alysiella*, CDC group EF-4, and CDC group M-5. *Veillonella* are Gram-negative cocci that are the anaerobic counterpart of *Neisseria*. These non-motile diplococci are part of the normal flora of the mouth.

The pathogenic bacteria in the Gram-negative aerobic cocci group include *Neisseria, Moraxella (Branhamella)*, and the *Acinetobacter*. The genus *Neisseria* includes two important human pathogens, *Neisseria gonorrhoeae* (urethritis, cervicitis, salpingitis, proctitis, pharyngitis, conjunctivitis, pharyngitis, pelvic inflammatory disease, arthritis, disseminated disease) and *Neisseria meningitides*(meningitis, septicemia, pneumonia, arthritis, urethritis). Other Gram-negative aerobic cocci that were previously considered harmless include *Moraxella (Branhamella) catarrhalis* (bronchitis and bronchopneumonia in patients with chronic pulmonary disease, sinusitis, otitis media) has recently been shown to be an common cause of human infections.

The EspA polypeptides of the invention can also be used to produce antibodies which are immunoreactive or bind to epitopes of the EspA polypeptides. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen containing fragments of the

protein by methods well known in the art (Kohler, *et al.*, *Nature*, 256:495, 1975; *Current Protocols in Molecular Biology*, Ausubel, *et al.*, ed., 1989).

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, Fab', $F(ab')_2$, and Fv that can bind the epitope. These antibody fragments retain some ability selectively to bind with its antigen or receptor and are defined as follows:

(1) Fab, the fragment that contains a monovalent antigen-binding fragment of an antibody molecule can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and part of one heavy chain;

(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and part of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $(Fab')_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody, defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable peptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (*See, for example,* Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (current edition), incorporated herein by reference).

An epitope is any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

If needed, polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the peptide or a peptide to which the antibodies are raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (*See, e.g.*, Coligan, *et al.*, Unit 9, *Current Protocols in Immunology*, Wiley Interscience, current edition, incorporated by reference).

The invention also provides peptide epitopes for use in designing *espA* specific nucleotide probes or anti-EspA antibodies. Such probes or antibodies can be used to identify proteins or genes that may be involved in the virulence of other pathogens, including but not limited to polypeptides or polynucleotides from Gram-negative bacteria.

The antibodies of the invention, including polyclonal and monoclonal antibodies, chimeric antibodies, single chain antibodies and the like, have with the ability to bind with high immunospecificity to the EspA proteins, peptides or nucleotide sequences of the invention, or fragments thereof. These antibodies can be unlabeled or suitably labeled. Antibodies of the invention can be used for affinity purification of EspA for example. Antibodies of the invention may be employed in known immunological procedures for qualitative or quantitative detection of these proteins or peptides in cells, tissue samples, sample preparations or fluids. Antibodies of the invention may be employed in known immunological procedures for qualitative or quantitative detection of the nucleotide sequences or portions thereof.

The invention provides a method for detecting EspA polypeptide in a sample, including contacting a sample from a subject with an antibody to EspA polypeptide; and

detecting binding of the antibody to EspA polypeptide. Binding is indicative of the presence of EspA polypeptide in the sample. As used herein, the term "sample" includes material derived from a mammalian or human subject or other animal. Such samples include but are not limited to hair, skin samples, tissue sample, cultured cells, cultured cell media, and biological fluids. For example, EspA polypeptide can be detected in HeLa cell (*e.g.*, human) culture.

As used herein, the term "tissue" refers to a mass of connected cells (*e.g.*, CNS tissue, neural tissue, or eye tissue) derived from a human or other animal and includes the connecting material and the liquid material in association with the cells. For example, rabbit enteropathogenic *E. coli* can be found in the stomach, cecum and colon of rabbits. As used herein, the term "biological fluid" refers to liquid material derived from a human or other animal. Such biological fluids include but are not limited to blood, plasma, serum, serum derivatives, bile, phlegm, saliva, sweat, amniotic fluid, and cerebrospinal fluid (CSF), such as lumbar or ventricular CSF.

As used herein, the term "sample" also includes solutions containing the isolated polypeptide, media into which the polypeptide has been secreted, and media containing host cells which produce the EspA polypeptide. For example, a sample may be a protein samples which is to be resolved by SDS-PAGE and transferred to nitrocellulose for Western immunoblot analysis. The quantity of sample required to obtain a reaction may be determined by one skilled in the art by standard laboratory techniques. The optimal quantity of sample may be determined by serial dilution.

In one embodiment, the presence of EspA polypeptide in the sample is indicative of infection by enteropathogenic *E. coli*. In another embodiment, the presence of EspA polypeptide in the sample is indicative of infection by enterohemorrhagic *E. coli*.

Proteins, protein fragments, and synthetic peptides of the invention are projected to have numerous uses including prognostic, therapeutic, diagnostic or drug design applications. Proteins, protein fragments, and synthetic peptides of the invention

will provide the basis for preparation of monoclonal and polyclonal antibodies specifically immunoreactive with the proteins of the invention. In one embodiment, the invention provides a method of immunizing a host susceptible to disease caused by EspA-producing *E. coli*, by administering to a host with the polypeptide of claim 1; and inducing a protective immune response in the host to EspA polypeptide. The infection of the host by EspA-producing organism is thereby prevented. In a more specific embodiment, the EspA-producing organism is an *E. coli* strain. In an even more specific embodiment, the *E. coli* strain is either enteropathogenic or enterohemorrhagic *E. coli*.

In another embodiment, the invention provides a method of ameliorating disease caused by EspA-producing organism, by immunizing a host with EspA polypeptide and inducing an immune response in the host to the EspA polypeptide. In a more specific embodiment, the EspA-producing organism is an *E. coli* strain. In an even more specific embodiment, the *E. coli* strain is either enteropathogenic or enterohemorrhagic *E. coli*. The invention provides a method for detecting *espA* polynucleotide in a sample, by contacting a sample suspected of containing *espA* polynucleotide with a nucleic acid probe that hybridizes to *espA* polynucleotide; and detecting hybridization of the probe with *espA* polynucleotide. The detection of hybridization is indicative of *espA* polynucleotide in the sample.

In another embodiment, the invention provides an organism with a mutated *espA* gene. Preferred organisms in which an *espA* gene may be mutated include but are not limited to bacteria. Among the bacteria in which an *espA* gene may be mutated are *E. coli*. Among the *E. coli* in which an *espA* gene may be mutated are enteropathogenic and enterohemorrhagic *E. coli*.

The invention provides a recombinant method for producing *espA* polynucleotide, including inserting a nucleic acid encoding a selectable marker into the polynucleotide encoding EspA polypeptide. The resulting polynucleotide encodes a recombinant EspA polypeptide containing the selectable marker. For example, a

selectable marker may be a herpes simplex virus (HSV) tag, for which there are commercially available antibodies.

The invention provides a recombinant method for producing EspA polypeptide, by growing a host cell containing a polynucleotide encoding EspA polypeptide under conditions which allow expression and secretion of EspA polypeptide; and isolating the polypeptide. Methods of producing polypeptides and peptides recombinantly are within the scope of this invention. As used herein, the term "conditions which allow expression and secretion" refers to suitable conditions such that the nucleic acid is transcribed and translated and isolating the polypeptide so produced. The polypeptide produced may be a protein secreted into the media. Media includes a fluid, substance or organism where microbial growth can occur or where microbes can exist. Such environments can be, for example, animal tissue or bodily fluids, water and other liquids, food, food products or food extracts, and certain inanimate objects. For example, microbes may grow in Luria-Bertani (LB) media. It is not necessary that the environment promote the growth of the microbe, only that it permits its subsistence.

The invention provides a method to identify a compound which inhibits bacterial type III secretion systems, by introducing the polynucleotide encoding a selectable marker into bacteria having a bacterial type III secretion system; growing the bacteria under conditions which allow growth of bacteria and secretion of the polypeptide encoded by the polynucleotide; contacting a compound suspected of inhibiting the bacterial type III secretion system with the bacteria; inducing the expression of the polypeptide; and detecting the secretion of the polypeptide. In the practice of the method, a lack of secretion is indicative of the inhibition of bacterial type III secretion systems. As used in this invention, the term "type III secretion" and "type III secretion" pathway refer to a specialized machinery to export molecules across a cell membrane. Exporting molecules across a cell membrane is a process critical for moving virulence factors to the surface where they can interact with host cell components. The type III secretion pathway uses adenosine triphosphate (ATP) as an energy source. The type III

secretion pathway is different than other secretion pathways found in Gram-negative bacteria, although it is homologous to flagella and filamentous phage assembly genes. It does not resemble any mammalian pathway. It is always associated with disease production. The virulence factors secreted by the type III secretion pathway vary between pathogens, although components of the type III secretion machinery are interchangeable, at least for *Salmonella*, *Shigella*, and *Yersinia*.

Furthermore, the polypeptide or nucleotide sequences of the invention can be used to identify compounds or compositions which interact (*e.g.*, bind) with them and affect their biological activity. Such effects include inhibition or stimulation of EspA activity or secretion.

The invention provides a method for producing a nonpathogenic organism, by generating a mutation in a polynucleotide encoding EspA polypeptide; inserting a nucleic acid sequence encoding a selectable marker into the site of the mutation; introducing the mutated *espA* polynucleotide into a chromosomal *espA* gene of an organism to produce a mutation in the chromosomal *esp*A gene; and selecting organisms having the mutation. As used herein, the term "mutation" refers to a change in the nucleotide sequence of a gene, in particular, the polynucleotide encoding EspA polypeptide. Mutations include mutations producing EspA polypeptide with a different amino acid sequence, missense mutations (including frame shift mutations), nonsense mutations (including knockout mutations), and recombinant genetic techniques which produce fusion proteins containing part of the EspA polypeptide. In one embodiment, the nucleic acid sequence encoding a selectable marker encodes resistance to kanamycin. For example, the *aph*A-3 cassette, encoding a gene for resistance to kanamycin (*kan*), may be cloned into the polynucleotides encoding the EspA polypeptide for selection of the mutated *espA* polynucleotide on kanamycin plates to produce a knockout mutation.

Preferred organisms in which to practice the invention include but are not limited to bacteria. In another embodiment, the organism which is used to generate a

mutation in a polynucleotide encoding EspA polypeptide is *E. coli*. Among the *E. coli* that may be transformed are enteropathogenic and enterohemorrhagic *E. coli*.

The invention provides a method of activating tyrosine kinase activity in a host cell by adding both mutant *esp*A-deficient organisms that express Eae polypeptide and mutant *eae*A-deficient organisms that express EspA polypeptide to a host cell and binding the bacteria to the host cell, thereby activating host cell tyrosine kinase activity in the cell. In one embodiment, the activation of host cell tyrosine kinase activity in the cell causes the tyrosine phosphorylation of a 90 kilodalton host membrane protein, Hp90, and fluxes of intracellular inositol phosphate (IP_3) and calcium. For example, an *eae*A mutant can be used to complement an *esp*A mutant for invasion when these two mutant strains were used to co-infect HeLa cells. The invention thus provides a useful scientific method to investigate pathogenesis by cell biology.

This invention includes a kit containing one or more antibodies of the invention as well as a nucleotide based kit. In one embodiment, the kit is useful for the detection of EspA polypeptide and is a carrier means compartmentalized to receive in close confinement a container containing an antibody which binds to EspA polypeptide. As used herein, a "container means" includes vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. In one embodiment, the antibody which binds to EspA polypeptide is detectably labeled. In a more specific embodiment, the label is selected from the group consisting of radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

In another embodiment, the kit is useful for the detection of an *esp*A polynucleotide and is a carrier means compartmentalized to receive in close confinement a container containing the nucleic acid probe that hybridizes to *esp*A polynucleotide. In one embodiment, nucleic acid probe that hybridizes to *esp*A polynucleotide is detectably labeled. In a more specific embodiment, the label is selected from the group consisting of

radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

Since EspA is a secreted protein, it is useful as a fusion partner for cloning and expressing other peptides and proteins. For example, EspA fused to a protein of interest is recombinantly produced in a host cell, *e.g.*, *E. coli*, and the fusion protein is secreted into the culture media in which the transformed host is grown. The fusion protein can be isolated by anti-EspA antibodies followed by cleavage of EspA from the peptide or protein of interest. ELISA or other immunoaffinity methods can be used to identify the EspA fusion protein. The invention provides a method of producing an EspA fusion protein including growing a host cell containing a polynucleotide encoding EspA operably linked to a polynucleotide encoding a polypeptide or peptide of interest under conditions which allow expression and secretion of the fusion polypeptides and isolating the fusion polypeptide. The term "operably linked or associated" refers to functional linkage between a promoter sequence and the structural gene or genes in the case of a fusion protein, regulated by the promoter nucleic acid sequence. The operably linked promoter controls the expression of the polypeptide encoded by the structural gene (*e.g.*, the fusion protein).

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be utilized.

Example 1

DNA Sequence Analysis of the Enteropathogenic *E. coli espA* Gene

The purpose of this Example is to characterize *espA*, a gene responsible for the attaching and effacing activity of enteropathogenic *E. coli*. The *espA* gene encodes the 25 kilodalton secreted protein and is located on the enteropathogenic *E. coli* genome

in the Locus of Enterocyte Effacement between *eae*A and *esp*B, two loci required for intimate adherence.

The DNA sequence of the enteropathogenic *E. coli* Locus of Enterocyte Effacement between *eae*A and *esp*B was determined. DNA sequencing was performed as follows: The SalI-BglII DNA fragment of the Locus of Enterocyte Effacement spanning from within *eae*A to upstream (5') of *esp*B was cloned into the commercially available plasmid pBluescript to create the plasmid pLCL109. A series of nested DNA deletions was made from the end of the plasmid pLCL109 closer to the *eae*B gene. These DNA deletions of plasmid pLCL109 were used as templates to determine the nucleotide sequence of both strands of DNA using oligonucleotide primers synthesized as needed, [a-^{35}S]dATP, and the Sequenase enzyme. DNA sequence data were analyzed with the package developed by the Genetics Computer Group of the University of Wisconsin.

Analysis of the DNA sequence showed three open reading frames. The amino acid sequence predicted by the DNA sequence at the 5' end of the second open reading frame (*esp*A) was identical to the amino-terminal sequence of a protein with an M_R of approximately 25 kalton secreted by enteropathogenic *E. coli*. No proteins with similar sequences were detected in a search of the Genbank database using the TFASTA. Therefore the *esp*A gene encodes the secreted enteropathogenic *E. coli* protein.

The predicted molecular weight of the entire protein encoded by *esp*A is 20,468 daltons (Fig. 1). No leader sequence precedes the amino-terminus of the reported secreted product. A strong consensus ribosome binding site ends seven nucleotides prior to the start codon. Eleven of the first nineteen residues at the amino-terminus were predicted to be serine or threonine.

Example 2

Construction of a Mutation in an *espA* Gene on a Plasmid and on a Chromosome.

The purpose of this Example was to construct a mutation in an *espA* gene on the chromosome of an organism. The purpose of this Example was to construct a mutation in an *espA* gene on a plasmid. The plasmid can then be used to create mutations in the *espA* gene of other organisms. Plasmids with a nonpolar mutation in *espA* gene were constructed. Furthermore, a mutant bacterial strain with a nonpolar mutation in its chromosomal *espA* gene was generated.

An *espA* gene with a nonpolar mutation is described in FIGURE 2. The use of the polymerase chain reaction (PCR) generated a deletion with restriction sites that allow an *aphA*-3 cassette, encoding resistance to kanamycin, to be cloned into the deleted region. This *aphA*-3 cassette is preceded (5') by translation stop codons in all three reading frames and immediately followed (3') by a consensus ribosome binding site and a start codon. The insertion into the *espA* gene was engineered to retain the reading frame of the 3' end of *espA* and to therefore allow unaffected transcription and translation of downstream (3') genes. DNA sequencing confirmed the reading frame of the mutation.

The construction of a nonpolar mutation in the *espA* gene on a plasmid was performed by the polymerase chain reaction as follows: The PCR template was plasmid pLCL114, containing the *ClaI- BglII* fragment of pLCL109 cloned into pBluescript. Two pairs of primers were used, the universal primer with Donne-99 and the reverse primer with Donne-100. Oligonucleotides Donne-99 and Donne-100 are nucleotides 4157 through 4140 and 4297 through 4324 of the SalI-BglII fragment, respectively. For cloning purposes, an *NruI* restriction site was engineered into the 5' end of both Donne-99 and Donne-100. Oligonucleotides were constructed at the Biopolymer Laboratory of the University of Maryland at Baltimore. PCR was performed on 50μL samples in a minicyler. The PCR reaction was thirty cycles of DNA denaturation at 94°C for one minute, annealing to the primers at 55°C for two minutes, and polynucleotide extension at 72°C for three minutes. The two resulting amplified fragments amplified

were each cloned into the commercially available plasmid pCRscript to create pLCl119 and pLCL120, respectively. The insert of pLCL120 was then cloned into pLCL119 using SalI and NruI to create pLCL121 containing the desired deletion. The 850 base pair *aphA*-3 kanamycin resistance cassette flanked by *Sma*I sites was then inserted into the *Nru*I site of pLCL121.

The mutated *esp*A allele was cloned in the positive-selection suicide vector pCVD442 and introduced into wild-type enteropathogenic *E. coli* strain E2348/69 by allelic exchange. A *esp*A mutant bacterial strain was constructed as follows: The SalI - SacI fragment from the plasmid with the *aphA*-3 kanamycin resistance which contained the interrupted *esp*A gene was cloned into positive-selection suicide vector pCVD442 in DH5*αpir* for introduction into E2348/69 by triparental conjugation or by electroporation in 0.1 cm cuvettes with an *E. coli* pulser set at 1.8 kV.

An *esp*A mutant was selected on modified LB kanamycin plates. The resulting enteropathogenic *E. coli* mutant strain, UMD872, was resistant to sucrose and kanamycin, and sensitive to ampicillin. PCR amplification using the two primers flanking the mutation, Donne-52 and Donne-73, confirmed the construction of the *esp*A mutation. Bacteria were stored at -70°C in 50% LB broth/50% glycerol (vol/vol) and grown on LB agar plates or LB broth with chloramphenicol (20μg/ml), ampicillin (200 μg/ml), nalidixic acid (50 μg/ml), or kanamycin (50 μg/ml) added as needed.

Example 3

Disruption of the enteropathogenic *E. coli esp*A Gene Abolishes Secretion of the EspA Protein.

The purpose of this Example was to identify the protein encoded by the *esp*A gene. A comparison of the nino-terminal sequence data of the EspA protein agreed with the translated*esp*A gene sequence. To confirm this result, the *esp*A deletion mutant, UMD872, was grown in radiolabeled tissue culture media. The deletion of the *esp*A gene results in the loss of the 25 kilodalton radiolabeled secreted protein. This result was

confirmed by immunoprecipitation using an anti-EPEC antisera which reacts with the secreted EspA protein. Western analysis using anti-EPEC antisera showed no 25 kilodalton secreted protein. Secretion of the EspA protein was restored when the *espA* deficient strain was transformed a plasmid (pMSD2) with an intact *esp*A. The increased production by the bacteria of EspA protein encoded by the plasmid reduced the secretion of the other proteins by the type III secretion pathway.

Example 4

Enteropathogenic *E. coli* EspA Is Required for Invasion of Cultured Epithelial Cells

The purpose of this Example was to examine whether the enteropathogenic *E. coli* EspA protein is involved in epithelial cell invasion. EspA protein is needed for triggering the host signal transduction pathway and invasion of host cells.

Monolayers of an epithelial cancer cell (HeLa) were infected for three hours with either parental wild-type or *esp*A mutant enteropathogenic *E. coli* strains. The number of adherent and intracellular (*i.e.*, invasive) enteropathogenic *E. coli* was determined. The absolute number of bacteria adherent to HeLa cells varied between strains, according to the different growth rates between the mutant and parental enteropathogenic *E. coli* strains. While the *esp*A mutant strain UMD872 adheres efficiently to epithelial cells, it is deficient for invasion. However, UMD872 invaded HeLa monolayers at near wild-type levels when the *esp*A gene was genetically complemented by the plasmid pMSD2, which encodes an intact *esp*A gene.

Co-infection experiments of HeLa monolayers were carried out to determine whether the invasion defective behavior of bacterial strains with mutations in either *esp*A, *esp*B, and *eae*A could genetically complement each other in *trans* to mediate subsequent invasion. The *eae*A bacterial mutant strain activates signaling but lacks intimate adherence. Signaling mediated by an *eae*A mutant strain allows an *esp*B deficient strain to enter epithelial cells, but not the converse. The *eae*A mutant complemented an *esp*A mutant for invasion when these two mutant strains were used to

co-infect HeLa cells, but there was no increase in invasion of the *eae*A mutant strain. Indeed, the signaling induced by the more adherent *eae*A mutant lead to the increased uptake of the *esp*A mutant. Co-infection with *esp*A and *esp*B deleted strains did not enhance invasion of either strain, showing that *esp*A and *esp*B do not complement each other.

Co-infection experiments demonstrated that like *esp*B, the *esp*A mutant behavior was complemented by *eae*A but not the reverse. Indeed, the signaling generated by the more adherent *eae*A mutant lead to the increased uptake of the intimin expressing *esp*A mutant. In contrast, neither the *esp*A nor the *esp*B mutant strain could complement each other, implying that both proteins may act together at the same step to induce epithelial signaling.

Example 5

EspA is Essential to Induce Signal Transduction Events in Epithelial Cells.

The purpose of this Example was to determine whether EspA is essential to induce signal transduction events in epithelial cells. Enteropathogenic *E. coli* induce tyrosine phosphorylation of a host cell 90 kilodalton membrane protein and subsequent accumulation of phosphorylated proteins, actin, and other cytoskeletal components beneath adherent bacteria. The ability of an *esp*A mutant defective for invasion to induce these two signaling events in mammalian cells was examined. Unlike wild-type enteropathogenic *E. coli*, the *esp*A mutant strain UMD872 was unable to induce phosphorylation of host Hp90. The ability to induce this phosphorylation event was restored by a plasmid (pMSD2) that encodes the EspA protein.

The adherence and invasion assays were performed as follows: 10^5 HeLa cells grown in DMEM were infected with the various enteropathogenic *E. coli* strains (m.o.i. 1:100) for three hours. HeLa cells were grown at 37°C with 5% CO_2 in Dulbecco's Minimal Eagles Medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. Monolayers were washed thrice in phosphate-buffered saline before lysing in

1% Triton (vol/vol) in phosphate-buffered saline and plating out serial dilution on LB agar plates. For invasion assays the washed monolayers were incubated with gentamicin (100μg/ml) for one hour to kill external bacteria before washing, lysing and plating out.

HeLa cell monolayers were infected for three hours with wild-type or mutant enteropathogenic *E. coli* strains. The epithelial Triton X-100 soluble and insoluble proteins of HeLa cells were isolated. Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose prior to probing with anti-phosphotyrosine specific antibodies.

The isolation of enteropathogenic *E. coli*-secreted proteins and HeLa cellular proteins was accomplished as follows: Tissue culture plates were seeded overnight with 10^6 HeLa cells. Before infection, the media was replaced with DMEM minus methionine/cysteine containing cycloheximide (100 μg/ml). HeLa cells were grown at 37°C with 5% CO_2 in DMEM supplemented with 10% (vol/vol) fetal calf serum. Enteropathogenic *E. coli* was added (m.o.i. 100:1) and incubated for 2.5 hours at 37°C in 5% CO_2 incubator before adding 200 μg/ml ^{35}S cysteine/methionine for 30 minutes. The culture supernatant was removed and the bacteria pelleted by centrifugation (18,000x g, 10 minutes). The supernatant secreted proteins were precipitated by the addition of ice cold trichloroacetic acid (10% vol/vol) and incubated on ice for 60 minutes. Proteins were pelleted by centrifugation as above and resuspended in Laemelli sample buffer. Samples were resolved by 12% SDS-PAGE and protein profiles examined by autoradiography or transferred to nitrocellulose prior to probing with anti-EPEC antibodies.

All enteropathogenic *E. coli* strains exhibited a tyrosine phosphorylated 85 kilodalton protein Ep85 in the insoluble fraction, confirming the presence of the enteropathogenic *E. coli* strains on the monolayer.

HeLa phosphotyrosine proteins were analyzed as follows: Infected HeLa monolayers were washed thrice with ice cold phosphate-buffered saline prior to lysis in 1% Triton X-100 in the presence of protease inhibitors. The Triton insoluble and soluble

fraction were isolated, resuspended in Laemelli sample buffer, and analyzed for the presence of phosphotyrosine proteins by Western immunoblot analysis with anti-phosphotyrosine antibodies.

Examination of infected HeLa cells by immunofluorescence microscopy with fluorescently labeled anti-phosphotyrosine antibodies or rhodamine-phalloidin showed that, unlike wild-type parental enteropathogenic *E. coli*, the *esp*A mutant did not accumulate tyrosine phosphorylated proteins or cytoskeletal actin beneath the adherent microcolonies. However, phosphotyrosine and actin accumulation could be restored by using a strain carrying the *esp*A gene on the plasmid pMSD2.

Immunofluorescence microscopy was performed as follows: HeLa cells which were seeded on round glass cover slips were infected with enteropathogenic *E. coli* or a mutant strain for three hours. The monolayers were then washed and fixed in 2.5% paraformaldehyde prior to staining for filamentous actin (using phallodin-rhodamine) or with anti-phosphotyrosine antibodies with an appropriate secondary fluorescein conjugated antibody.

Example 6

Characterization of Rabbit Enteropathogenic *E. coli* (RDEC-1) Secreted Virulence Proteins, EspA and EspB

The purpose of this Example was to investigate the structure of EspA and EspB in rabbit enteropathogenic *E. coli* (RDEC-1). The *esp*A and *esp*A genes were cloned and their sequences were compared to those of enteropathogenic *E. coli* (EPEC). The EspA protein showed some similarity (88.5% identity). The EspB protein was heterogeneous in internal regions (69.8% id- ity), but was identical to one strain of enterohemorrhagic *E. coli* (EHEC).

Cloning and sequence analysis of *esp*A and *esp*B genes was done as follows: The DNA fragment encoding RDEC-1 *esp*A and *esp*B was obtained by PCR from RDEC-1 chromosomal DNA using primers derived from the published sequence of

enteropathogenic *E. coli*. Vent DNA polymerase was used for PCR to amplify chromosomal DNA from RDEC-1 and enteropathogenic *E. coli* strains. The PCR reaction was carried out for thirty cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and elongation at 72°C for two minutes. The resulting 4.3 kilobase pair product was ligated into the commercially available plasmid pBluescript and both strands were sequenced. DNA sequencing was done as follows: The 4.3 kilobase pair DNA fragment encoding the *esp*A and *esp*B genes was amplified by PCR using the primers AA01(+) and MS11(-), and RDEC-1 chromosomal DNA as the DNA template. The resulting blunt end fragment was digested with SalI and cloned into the SalI-SmaI site of the commercially available plasmid pBluescript-II SK (+). The DNA sequence of RDEC-1 *esp*A and both strands using the commercially available Taq DyeDeoxy kit. (Figure 3)

Two open reading frames were found in the cloned region and these DNA sequences were similar to enteropathogenic *E. coli esp*A and *esp*B. The predicted molecular weight of RDEC-1 EspA (192 amino acids) was 23,533 dalton, and RDEC-1 EspB (314 amino acids) was 33,219 dalton. RDEC-1 EspA was somewhat similar to that of enteropathogenic *E. coli* with 88.5% identity (Figures 4A and 4B).

In an unexpected result, RDEC-1 EspB protein was identical to the recently reported EspB from enterohemorrhagic *E. coli* strain 413/89-1 serotype 026, which was originally isolated from a calf and also isolated from patients with hemolytic uraemic syndrome, although two nucleotide differences occurred at positions 12 (T to C) and 729 base pair (G to T). Furthermore, RDEC-1 EspB showed 70.3% enc. 69.8% identity respectively to that of enterohemorrhagic *E. coli* serotype 0157 and enteropathogenic *E. coli* strains. Small sequence deletions were found in RDEC-1 and enterohemorrhagic *E. coli* (serotype 026 and 0157) EspB at the same positions when compared to the enteropathogenic *E. coli* sequences (Figures 4A-C).

These results show that RDEC-1 encodes *esp*A and *esp*B genes, and that the predicted EspA polypeptide is highly conserved in RDEC-1 and enteropathogenic *E. coli*.

However EspB is more similar to that of enterohemorrhagic *E. coli* rather than enteropathogenic *E. coli*. An open reading frame downstream (3') from *esp*A showed similarity to enteropathogenic *E. coli* EspD, a secreted protein that modulates EspB secretion and is needed for triggering of host signal transduction pathways (EMBL GenBank data, Accession No. Y09228). These results show that *esp*D is also located between *esp*A and *esp*B in RDEC-1.

Example 7

Characterization of RDEC-1 EspA and EspB

The purpose of this Example was to investigate the function of EspA and EspB in rabbit enteropathogenic *E. coli* (RDEC-1). Mutations in RDEC-1 *esp*A and *esp*B revealed that the RDEC-1 gene products are essential for triggering of host signal transduction pathways and invasion into HeLa cells. Complementation with plasmids containing enteropathogenic *E. coli esp*A and *esp*B into RDEC-1 mutant strains demonstrated that they were functionally interchangeable, although the enteropathogenic *E. coli* proteins mediated higher levels of invasion. Furthermore, maximal expression of RDEC-1 and enteropathogenic *E. coli* secreted proteins occurred at their respective host's body temperatures, which may contribute to lack of enteropathogenic *E. coli* infectivity in rabbits.

To confirm the role of RDEC-1 *esp*A and espB in host epithelial signal transduction pathways, a non-polar stop codon mutation was engineered into *esp*A and espB. Two suicide vectors were constructed and introduced into the RDEC-1 wild type strain by back conjugation. The resulting mutant strains, AAF001ΔA ($EspA^-$), AAF001ΔB ($EspB^-$), and double mutant strain AAF001ΔAB ($EspA^-/EspB^-$) were confirmed by BglII digestion.

The construction of the non-polar stop codon mutations in RDEC-1 *esp*A and *esp*B genes was performed as follows: The 2.7 kilobase pair DNA fragment of the Locus of Enterocyte Effacement encoding the *esp*A and *esp*B genes was amplified by PCR

using the primers BK25(+) and MSII(-), and pORF123B as the DNA template. The resulting blunt end fragment was digested with EcoRI and cloned into the EcoRI-SmaI site of pBluescript II SK(+) vector to obtain pORF23B. A 1.1 kilobase pair EcoRI-BglII DNA fragment from pORF23B containing *esp*A was cloned into the EcoRI-BamHI site of pBluescript II SK(+) to obtain pORF23.

To construct a non-polar mutation in *esp*A, inverse PCR was carried out using the ΔespA(+) and ΔespA(-) primers which contain a BglII restriction site and a stop codon using circular pORF23 as a DNA template. The PCR product was then blunt end ligated to obtain pORF23Δ. The resulting plasmid contained a stop codon and a BglII site 235 base pair downstream (3') from the *esp*A start codon, which was confirmed by DNA sequencing. The 1.1 kilobase pair SalI-SacI DNA fragment containing the *esp*A mutation from pORF23A was inserted into the same sites of the suicide vector pCVD442, which contains the sacB gene for positive selection and an ampicillin resistance gene, to obtain pAA23Δ. The resulting plasmid was introduced into E. coli SM10λpir and back conjugated into RDEC-1 harboring pACYC184.

For the non-polar mutation in *esp*B, inverse PCR was carried out using the ΔespB(+) and ΔespB(-) primers, and pBxb as a DNA template. pBxb contains the 1.4 kilobase pair XbaI fragment from pORF23B encoding *esp*B cloned into the pBluescript vector. The resulting PCR product was self-ligated to obtain pBxbΔ that contained a stop codon and a BglII site introduced by the ΔespB(-) and ΔespB(+) primers. The resulting *esp* gene in pBXbΔ was deleted by 250 base pair, starting 154 base pair downstream (3') of the *esp*B start codon. The 1.1 kilobase pair SalI-SacI site DNA fragment containing the *esp*B mutation from pBxbA was inserted into the same site of the pCVD442 to obtain pAABxbΔ. The resulting plasmid was transformed into *E. coli* SM10λpir and back conjugated into RDEC-1 harboring pACYC184. To establish double mutations in *esp*A and *esp*B, pAABxbΔ was introduced into AAF001ΔA (EspA$^-$) strain. Three RDEC-1 non-polar mutant strains were confirmed by their phenotypes which maintain resistance to sucrose and chloramphenicol, and sensitivity to ampicillin. To confirm the stop codon

insertions in *esp*A and *esp*B, chromosomal DNA was prepared from each mutant strain and PCR was performed with primers encompassing the esp genes. The resulting PCR products were digested with BglII to confirm the presence of this engineered restriction site. The mutant strains containing the stop codon in *esp*A or/and *esp*B were designated as AAF001ΔA (EspA$^-$), AAF001ΔB (EspB$^-$) and AAF001ΔAB (EspA$^-$/EspB$^-$), respectively.

Mutations back to wild type ("back mutation") were made to confirm that suicide vectors do not affect respective flanking region or other loci. Two back mutant strains were obtained by bans conjugation of the suicide vectors pAA23 and pAABxb into AAF001ΔA (EspA$^-$) and AAF001ΔB (EspB$^-$) strains. The resulting back mutant strains, AAF003 and AAF004, were confirmed by PCR and BglII digestion. The construction of back mutations in EspA and EspB strains was done as follows: The 1.1 kilobase pair SalI-SacI DNA fragment from pORF23 containing *esp*A was inserted into the SalI-SacI sites of pCVD442 to obtain pAA23. The 1.4 kilobase pair SalI-SacI fragment of pBxb was inserted into the SalI-SacI site of pCDD442 to obtain pAAFBxb. pAA23 and pAABxb were introduced into SMλpir and bans conjugated into AAF001AA and AAF001AB. The resulting back mutant strains were confirmed as described above and designated as AAF002 (EspA+) and AAF003 (EspB+).

The cloning of the enteropathogenic *E. coli esp*A and *esp*B genes was done as follows: The 2.8 kilobase pair DNA fragment encoding *esp*A and *esp*B was amplified by PCR using the primers EespA(+) and EespB(-) with enteropathogenic *E. coli* 2348/69 chromosomal DNA as the template. This fragment was digested with BamHI and SalI and introduced into the BamHI-SalI site of the low-copy vector pMW118 under control of the *lacZ* promoter, to obtain pMWespAB. pMWespAB was digested with BglII which has a restriction site in *esp*D open reading frame, blunt ended with Klenow fragment, and then self-ligated to obtain pMW6espD. pMWespAB was also digested with BglII and BamHI, and then self ligated to obtain pMWespB. The PMWespAB was digested with BglII-SalI, and filled with Klenow Fragment, then self-ligated to obtain pMWespA.

The secretion profile of RDEC-1 and its mutant strains in tissue culture media was analyzed. Enteropathogenic *E. coli* secretes five proteins, 110 kilodalton (EspC), 40 kilodalton, 39 kilodalton, 37 kilodalton (EspB), and 25 kilodalton (EspA) in culture media. RDEC-1 showed a similar secretion profile, except it did not secrete a protein equivalent to EspC. EspC is not required for enteropathogenic *E. coli* induction of host signal transduction pathways. Although two secreted proteins (40 and 39 kilodalton) were difficult to resolve, these proteins could be resolved using different conditions of SDS-PAGE. RDEC-1 secreted two proteins with similar mobility to enteropathogenic *E. coli* EspA and EspB.

RDEC-1 secreted proteins were prepared as follows: Bacterial overnight cultures were diluted 1:100 into DMEM and incubated to an optical density of 1.0 at 600 nm (OD600). For RDEC-1 mutant strains containing enteropathogenic *E. coli espA* and *espB* recombinant plasmids, isopropylthiogalactoside (IPTG) was added in DMEM to induce transcription. Bacteria were removed by centrifugation (18,000 x g, 10 minutes) and the supernatant precipitated by addition of 10% ice-cold trichloroacetic acid, and incubated on ice for one hour. After centrifugation, the pellets were resuspended in Laemmli sample buffer and analyzed by 12% SDS-PAGE.

Both EspA and EspB proteins cross-react to anti-enteropathogenic *E. coli* EspA and anti-enteropathogenic *E. coli* EspB antisera in Western immunoblots, indicating that RDEC-1 also secretes EspA and EspB proteins. Rabbit polyclonal antibodies against enteropathogenic *E. coli* EspA and EspB were used in Western blots.

Mutant strains AAF001ΔA, AAF001ΔB, and AAF001ΔAB lack secretion of EspA, EspB, and EspA/EspB proteins, respectively, as judged by their secretion profile and Western blot analysis. EspB, whose gene is located downstream (3') from *espA*, was still secreted in the mutant strain AAF00ΔA (EspA$^-$), indicating that the stop codon insertion mutation does not affect downstream gene expression. These results also confirm that RDEC-1 EspA and EspB proteins are encoded by the sequences we

designated as *esp*A and *esp*B. Furthermore, the two back mutant strains AAF002 and AAF003, that were originally derived from AAF001ΔA (EspA$^-$) and AAF001ΔB (EspB$^-$), now expressed the parental secreted proteins indicating that each non-polar mutation in AAF001ΔA and AAF001ΔB is as predicted, and does not affect downstream genes and other loci. Although the mobilities of RDEC-1 EspA and EspB in SDS-PAGE were slightly faster than that of enteropathogenic *E. coli*, the calculated molecular masses of RDEC-1 EspA and EspB were greater than that of enteropathogenic *E. coli*.

The amount of the other secreted proteins were decreased in the EspA$^-$, EspB$^-$, EspA$^-$/EspB$^-$ strains when compared to wild type RDEC-1 strain. Furthermore, the decrease of detectable secretion of the 40 kilodalton and 39 kilodalton proteins in the EspA$^-$/EspB$^-$ strain is greater than that found in EspA$^-$ and EspB$^-$ strains. Secretion of enteropathogenic *E. coli* proteins, except EspC, are mediated by a type III secretion system encoded by the sep cluster. It is possible that truncation of EspA or EspB by inserting a stop codon may interfere with this secretion pathway or feedback regulation of this system, thereby affecting secretion of the other type III dependent secreted proteins.

Esp proteins are needed for triggering of the host signal transduction pathway. Enteropathogenic *E. coli* EspA and EspB proteins induce host signal transduction pathways resulting in accumulation of tyrosine phosphorylated proteins, cytoskeletal actin, and other components beneath the adherent bacteria. To determine whether RDEC-1 EspA and EspB trigger these events in HeLa cells, cytoskeletal actin add tyrosine-phosphorylated proteins were stained with rhodamine-phallodin and fluorescently labelled anti-phosphotyrosine antibody. Although the level of accumulation of cytoskeletal actin and tyrosine-phosphorylated protein beneath the attached RDEC-1 is lower than that of enteropathogenic *E. coli*, these behaviors were indistinguishable to that of enteropathogenic *E. coli*. By contrast, RDEC-1 EspA$^-$, EspB$^-$, and EspA$^-$/EspB$^-$ strains did not accumulate cytoskeletal act in or tyros ine-

phosphorylated proteins beneath the attached bacteria. However, the back mutant strains AAF003 and AAF004 accumulated these proteins similar to the parental strains.

When plasmids containing enteropathogenic *E. coli espA* or *espB* or both were introduced into the RDEC-1 EspA$^-$, EspB$^-$, and EspA$^-$/EspB$^-$ strains, the accumulation of cytoskeletal actin and tyrosinephosphorylated proteins was also restored. However, when the enteropathogenic *E. coli* EspA$^-$ strain, which still secretes EspB, was coinfected with RDEC-1 EspB, induction of host signal transduction events were not restored. Enteropathogenic *E. coli* EspB also did not complement RDEC-1 EspA in co-infection experiment. Therefore, functionally EspA and EspB are similar in RDEC-1 and enteropathogenic *E. coli* with respect to activating host signal transduction pathways, although both proteins need to be secreted by the same strain.

Tyrosine-phosphorylated Hp90 could be detected by immunoblotting when HeLa cells were infected with enteropathogenic *E. coli*. Tyrosine-phosphorylated Hp90 could not be detected with RDEC-1 infected cells, even though tyrosine phosphorylated proteins could be observed under adherent RDEC-1 bacterial cells by immunofluorescence.

Enterohemorrhagic *E. coli* does not induce tyrosine phosphorylation in HEp-2 and T84 cells as judged by immunofluorescence microscopy. The sequencing results showed that RDEC-1 EspB is more similar to that of enterohemorrhagic *E. coli* than enteropathogenic *E. coli*. These results in this Example show that the lower accumulation of tyrosine-phosphorylated proteins during RDEC-1 infection is due to lower adherence efficiency of RDEC-1 because of differences in adhesion levels or heterogeneity of Esp proteins or both.

Adherence and invasion ability. Enteropathogenic *E. coli* EspA and EspB are not only involved in triggering of host signal transduction pathways, but also necessary for invasion *in vitro*. In order to investigate the role of RDEC-1 EspA and EspB in adherence and invasion, RDEC-1 esp mutant strains were compared to that of RDEC-1. The adherence ability of EspA$^-$, EspB$^-$, and EspA$^-$/EspB$^-$ strains were similar to that of the

wild type RDEC-1 strain, indicating that adherence is independent of EspA and EspB expression. Although the invasive ability of wild type RDEC-1 was about ninety times lower than that of enteropathogenic *E. coli*, this ability was further decreased in the mutant strains EspA$^-$, EspB$^-$, and EspA$^-$/EspB$^-$. However, invasion was restored by back mutation strains AAF002 and AAF003. These results show that RDEC-1 invasion ability depends upon EspA and EspB.

To determine the ability of enteropathogenic *E. coli* EspA and EspB to complement the RDEC-1 mutants, various plasmids containing the enteropathogenic *E. coli esp*A and *esp*B genes were introduced into the RDEC-1 mutant strains, and invasion efficiencies compared to that of wild type RDEC-1 strain. Interestingly, the invasion levels of AAF001ΔAB (RDEC-1 EspA$^-$/EspB$^-$) harboring pMWespAB (EPEC EspA+/EspB+) was four times greater than that of wild type RDEC-1, even though the amount of secreted enteropathogenic *E. coli* EspA and EspB in AAF001ΔAB strain was lower than that normally found in RDEC-1. Therefore, the different invasion levels observed between RDEC-1 and enteropathogenic *E. coli* strains in HeLa cells can be attributed to the Esp proteins, and enteropathogenic *E. coli* EspA and EspB are more efficient at mediating invasion in this tissue culture model. Homology comparisons showed that EspA was highly conserved in RDEC-1 and enteropathogenic *E. coli*, but EspB was more heterogeneous, showing that the difference of invasive abilities between RDEC-1 and enteropathogenic *E. coli* may be due to the EspB protein. Interestingly, enterohemorrhagic *E. coli* 0157 adheres to, but does not invade into human ileocecal (HCT-8) epithelial cells. RDEC-1 EspB was more similar to that of enterohemorrhagic *E. coli* rather than enteropathogenic *E. coli*, perhaps emphasizing the role of EspB in invasion. These findings strongly support that Esp heterogeneity affects the invasive ability of enteropathogenic *E. coli*, RDEC-1, and enterohemorrhagic *E. coli*.

EspD mutant affects EspA and EspB secretion. Enteropathogenic *E. coli* contains an open reading frame, espD, located between *esp*A and *esp*B. To confirm the role of the *esp*D product in secretion, the plasmid pMWespD encoding enteropathogenic

E. coli espA, ΔespD (frame shift mutation at the BglII site), and *espD* was constructed and introduced into the RDEC-1 double mutant strain, AAF001AAB. The amount of enteropathogenic *E. coli* EspA and EspB secreted proteins in AAF001AAB [pMWespD] was lower than that in AAF001AAB [pMWespAB], which contains fragment encoding intact enteropathogenic *E. coli espA*, *espD*, and *espB* genes. Furthermore invasion ability was also decreased. These results show that disruption of *espD* affects secretion of enteropathogenic *E. coli* EspA and EspB proteins. In this Example, we showed that mutations in *espA* and/or *espB* also reduced the amount of the other secreted proteins, probably due to their truncated products. Secretion levels were more decreased in *espA* and *espB* double mutants when compared with *espA* or *espB* mutants. Thus, truncated enteropathogenic *E. coli* EspD may affect the secretion of EspA and EspB in AAF001AAB [pMW6espD] due to interference in type III secretion system. Whether or not EspD is directly involved in this secretion system is still unclear.

Enteropathogenic *E. coli* and RDEC-1 secreted proteins are tightly controlled by temperatures, which correspond to their relevant host body temperatures. Temperature regulates the expression of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* 413/89-1 secreted proteins. EspB expression was greatly increased when the temperature was shifted from 20°C to 37°C. Because EspA and EspB proteins are regulated by appropriate host body temperatures, wild type enteropathogenic *E. coli* and RDEC-1 strains were inoculated into DMEM and the secreted proteins were prepared following incubation at various temperatures, then analyzed by SDS-PAGE. Expression of enteropathogenic *E. coli* secreted proteins were visible at 33°C and reached maximal secretion level at 36°C. Expression was decreased at 39°C, and no secreted proteins were seen at 42°C. In contrast, maximal expression of RDEC-1 secreted proteins occurred at 39°C and these proteins were still expressed at 42°C. These results show that the maximal expression of Esp proteins in enteropathogenic *E. coli* and RDEC-1 are triggered by their relevant host's body temperature, human (37°C) and rabbit (39°C).

In conclusion, both proteins were needed to trigger host signal transduction pathways and invasion. Complementation experiments using enteropathogenic *E. coli* esp genes revealed that host signal transduction events triggered by RDEC-1 and enteropathogenic *E. coli* appear to be mediated by the same secreted proteins. Finally, optimal expression of RDEC-1 and enteropathogenic *E. coli* secreted proteins correlated with their natural host's body temperature. This explains their strict host specificity and the lack of enteropathogenic *E. coli* infection in rabbits or other animals. Animal infection studies using RDEC-1 *esp*A and *esp*B strains will provide information about the role of these secreted proteins in virulence, and may possibly be useful for vaccine studies.

Example 8

Two Rabbit Enteropathogenic *E. coli* (RDEC-1) Secreted Proteins, EspA and EspB, Are Virulence Factors

The purpose of this Example is to demonstrate the role of EspA and EspB proteins in pathogenesis. To investigate the role of these proteins in virulence, mutations in *esp*A and *esp*B were constructed in the rabbit enteropathogenic *E. coli* strain, RDEC-1.

RDEC-1 and its *esp*A and *esp*B mutant strains were inoculated by the orogastric route into young rabbits. Most RDEC-1 was found in the cecum and colon one week postinfection. However, the number of either mutant strain was greatly decreased in these tissues compared to the parent strain. RDEC-1 adhered specifically to the sacculus rotundas (follicle associated epithelium) and bacterial colonization was also observed in the cecum, indicating that the sacculus rotundas in the cecum is an important colonization site for this pathogen. The adherence levels of the EspA$^-$ and EspB$^-$ strains to the sacculus rotundas were 70 and 8000 times less than that of parent strain. These results show that the adherence ability and tissue tropism of RDEC-1 are dependent on the two Esp secreted proteins. Furthermore, EspB appears to play a more critical role

than EspA in bacterial colonization and pathogenesis. This is the first demonstration that the enteropathogenic *E. coli* secreted proteins, EspA and EspB, which are involved in triggering of host cell signal transduction pathways, are also needed for colonization and virulence.

Animal infections were performed as follows: Overnight bacterial cultures were collected by centrifugation and resuspended in one ml of phosphate-buffered saline. New Zealand white rabbits (weight 1.0 to 1.6 kg) were fasted overnight, then five ml of 2.5% sterile sodium bicarbonate and one ml of RDEC-1 or *espA* or *espB* strains ($2.5x10^{10}$) were inoculated into the stomach using orogastric tubes. The same dosage of bacteria was inoculated into each rabbit the following day.

Clinical assessments were performed as follows: Each rabbit was weighed daily and fecal shedding of bacteria were collected by rectal swabs and from stool pellets. Rectal swabs were rolled over one half of the surface of MacConkey plates containing nalidixic acid. Five stool pellets or same amount of liquid stool were collected from each rabbit and resuspended in three ml phosphate-buffered saline and 0.1 ml of each stool suspension was plated onto MacConkey plate containing nalidixic acid. The growth of nalidixic resistant colonies was scored as follows: O, no growth; 1, widely spaced colonies; 2, closely spaced colonies; 3, confluent growth of colonies.

Sampling and preparation of tissue were performed as follows: Tissues were excised immediately following sacrifice by intravenous injection of ketamine and overdosing with sodium phenobarbital.

The amount of bacterial colonization in intestinal tissues was assayed as follows: The intestinal segments (10 cm), except cecum, were doubly ligated at their proximal and distal ends, and dissected between the double ligated parts, then flushed with 10 ml of ice-cold phosphate-buffered saline. One gram of viscous contents from the cecum was added to 9 ml phosphate-buffered saline. The resulting phosphate-buffered saline suspensions were diluted and plated on MacConkey plates containing nalidixic acid.

The amount of bacterial adherence to intestinal tissues was assayed as follows: Tissue samples were excised using a nine mm diameter cork punch, washed three times with phosphate-buffered saline, added to two ml of ice-cold phosphate-buffered saline, and homogenized with a homogenizer, then serial diluted samples were plated onto MacConkey plates. The numbers of bacteria adherent to each tissue per square centimeter were calculated as follows: CFU/cm2 = the bacterial number/plate x dilution factor x 2 ml/~ 0.452

Example 9

Development of an Assay to Screen for Inhibitors of Bacterial Type III Secretion

The purpose of this Example is to provide an assay to screen for inhibitors of bacterial type III secretion.

A polynucleotide encoding the EspA polypeptide is fused to several well known molecules, including a HSV tag. The gene fusion is still secreted out of enteropathogenic *E. coli*. A plasmid contains the genetic region of *esp*A that encodes the amino-terminal portion of EspA (needed to mediate type III secretion) fused to a Herpes Simplex Virus (HSV) sequence that encodes a sequence tag to which commercial antibodies are available. This plasmid is transformed into a strain that contains an *esp*A mutation yet still secretes the other enteropathogenic *E. coli*-secreted proteins that use the type III secretion system. The supernatant of organisms containing these fusions is collected, added to an ELISA plate, followed by standard ELISA. A calorimetric readout indicates the fusion protein is secreted.

This plasmid is also transformed into a strain which is defective for type III secretion (*i.e.* a negative control). When the fusion protein is expressed in this strain, the fusion protein is expressed but not secreted. ELISA results with this mutant confirm that it is not secreted.

Thus an easy ELISA assay to look at secretion is provided. This assay is simple, and requires no special technology. It is also economical, because no expensive

reagents are needed. It is automated and used to screen reagents to identify inhibitors of bacterial type III secretion.

To assay for secretion, bacteria are grown standing overnight in tissue culture fluid in the presence of compounds to be tested. These conditions yield enteropathogenic *E. coli*-mediated secretion. The following day, bacteria are removed by centrifugation, and the supernatant placed into wells of a 96 well microtiter plate. A standard ELISA is performed on the supernatants. If the compound being tested is bactericidal, the bacteria do not grow overnight.

A polynucleotide encoding another polypeptide secreted by enteropathogenic *E. coli* is fused to several well known molecules. A polynucleotide encoding EspB polypeptide was fused a HSV tag, to which commercial antibodies are available.. The gene fusion was still secreted out of enteropathogenic *E. coli*. This plasmid was transformed into a strain that contained an *espA* mutation yet still secretes the other enteropathogenic *E. coli*-secreted proteins that use the type III secretion system. The supernatant of organisms containing these fusions is collected, added to an ELISA plate, followed by standard ELISA technology with, for example, anti-HSV antibodies. This screen was used to assay plant extracts from medically important plants. Dilutions of 1/200-1/1000 (about 250 µg/ml) are appropriate. Promising compounds are rescreened in the ELISA secretion assay to check for reproducibility.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. An isolated EspA polypeptide characterized by:
   a) being a secreted protein from enteropathogenic or enterohemorrhagic *E. coli*; and
   b) having a molecular weight of about 25 kilodaltons as determined by SDS-PAGE.

2. The polypeptide of claim 1, wherein the polypeptide has an amino acid sequence as set forth in SEQ ID NO:2.

3. The polypeptide of claim 1, wherein the polypeptide has an amino acid sequence as set forth in SEQ ID NO:4.

4. An isolated polynucleotide encoding the polypeptide of claim 1.

5. The polynucleotide of claim 4, wherein the sequence encodes the amino acid sequence set forth in SEQ ID NO: 2.

6. The polynucleotide of claim 5, wherein the sequence is selected from the group consisting of:

a) the nucleic acid sequence set forth in SEQ ID NO: 1;

b) the nucleic acid sequence set forth in SEQ ID NO: 1, wherein T is U;

c) nucleic acid sequences complementary to a); and

d) fragments of a) or b) that are at least 15 nucleotide bases in length and that hybridize under stringent conditions to genomic DNA which encodes the polypeptide set forth in SEQ ID NO: 2.

7. The polynucleotide of claim 4, wherein the sequence encodes the amino acid sequence set forth in SEQ ID NO: 4.

8. The polynucleotide of claim 7, wherein the sequence is selected from the group consisting of:

a) the nucleic acid sequence set forth in SEQ ID NO: 3;

b) the nucleic acid sequence set forth in SEQ ID NO: 3, wherein T is U;

c) nucleic acid sequences complementary to a); and

d) fragments of a) or b) that are at least 15 nucleotide bases in length and that hybridize under stringent conditions to genomic DNA which encodes the polypeptide set forth in SEQ ID NO: 4.

9. A vector containing the polynucleotide of claim 4.

10. A host cell containing the vector of claim 9.

11. An anti-EspA antibody which binds to the polypeptide of claim 1.

12. The antibody of claim 11, wherein the antibody is monoclonal.

13. The antibody of claim 11, wherein the antibody is polyclonal.

14. A method for detecting EspA polypeptide in a sample, comprising:
a) contacting the sample with the antibody of claim 11; and
b) detecting binding of the antibody of claim 11 to EspA polypeptide, wherein binding is indicative of the presence of EspA polypeptide in the sample.

15. The method of claim 14, wherein the sample is tissue.

16. The method of claim 14, wherein the sample is a biological fluid.

17. The method of claim 14, wherein the presence of EspA polypeptide in the sample is indicative of infection by enteropathogenic *E. coli*.

18. The method of claim 14, wherein the presence of EspA polypeptide in the sample is indicative of infection by enterohemorrhagic *E. coli*.

19. A method of immunizing a host susceptible to disease caused by EspA-producing *E. coli*, comprising:
a) administering to a host EspA polypeptide; and
b) inducing a protective immune response to EspA in the host, thereby preventing infection of the host by an EspA-producing organism.

20. The method of claim 19, wherein the EspA-producing organism is *E. coli*.

21. The method of claim 20, wherein the EspA-producing *E. coli*. is enteropathogenic *E. coli*.

22. The method of claim 20, wherein the EspA-producing *E. coli.* is enterohemorrhagic *E. coli.*

23. A method of ameliorating disease caused by EspA-producing organism, comprising:

a) immunizing a host with the polypeptide of claim 1; and

b) inducing an immune response in the host to the EspA polypeptide, thereby ameliorating disease caused by infection of the host by EspA-producing organism.

24. The method of claim 23, wherein the EspA-producing organism is *E. coli.*

25. The method of claim 23, wherein the EspA-producing *E. coli.* is enteropathogenic *E. coli.*

26. The method of claim 23, wherein the EspA-producing *E. coli.* is enterohemorrhagic *E. coli.*

27. A method for detecting *esp*A polynucleotide in a sample, comprising:

a) contacting a sample suspected of containing *esp*A polynucleotide with a nucleic acid probe that hybridizes to *esp*A polynucleotide; and

b) detecting hybridization of the probe with *esp*A polynucleotide, wherein the detection of hybridization is indicative of *esp*A polynucleotide in the sample.

28. The method of claim 27, wherein the nucleic acid probe is a polynucleotide of claim 4.

29. A recombinant method for producing *esp*A polynucleotide, comprising:
inserting a nucleic acid encoding a selectable marker into the polynucleotide of claim 4, such that the resulting polynucleotide encodes a recombinant EspA polypeptide containing the selectable marker.

30. A polynucleotide produced by the method of claim 29.

31. A host cell containing the polynucleotide of claim 30.

32. A recombinant method for producing EspA polypeptide, comprising:
a) growing a host cell containing a polynucleotide encoding EspA polypeptide under conditions which allow expression and secretion of EspA polypeptide; and
b) isolating the polypeptide.

33. A method to identify a compound which inhibits bacterial type III secretion systems, comprising:
a) introducing the polynucleotide of claim 29 into bacteria having a bacterial type III secretion system;
b) growing the bacteria under conditions which allow growth of bacteria and secretion of the polypeptide encoded by the polynucleotide;
c) contacting a compound suspected of inhibiting the bacterial type III secretion system with the bacteria;
d) inducing the expression of the polypeptide; and
e) detecting the secretion of the polypeptide; wherein a lack of secretion is indicative of the inhibition of bacterial type III secretion systems.

34. A method for producing a nonpathogenic organism, comprising:

a) generating a mutation in a polynucleotide encoding EspA polypeptide;

b) inserting a nucleic acid sequence encoding a selectable marker into the site of the mutation;

c) introducing the mutated *espA* polynucleotide of step b) into a chromosomal *espA* gene of an organism to produce a mutation in the chromosomal *esp*A gene; and

d) selecting organisms having the mutation.

35. The method of claim 34, wherein the nucleic acid sequence encoding a selectable marker encodes resistance to kanamycin.

36. The method of claim 34, wherein the organism is *E. coli*.

37. An organism with a mutated *esp*A gene produced by the method of claim 34.

38. A kit useful for the detection of EspA polypeptide comprising carrier means being compartmentalized to receive in close confinement therein one or more containers comprising a container containing an antibody which binds to EspA polypeptide.

39. The kit of claim 38, wherein the antibody is detectably labeled.

40. The kit of claim 39, wherein the label is selected from the group consisting of radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

41. A kit useful for the detection of an *espA* polynucleotide comprising carrier means being compartmentalized to receive in close confinement therein one or more containers comprising a container containing the nucleic acid probe that hybridizes to *espA* polynucleotide.

42. The kit of claim 41, wherein the probe is detectably labeled.

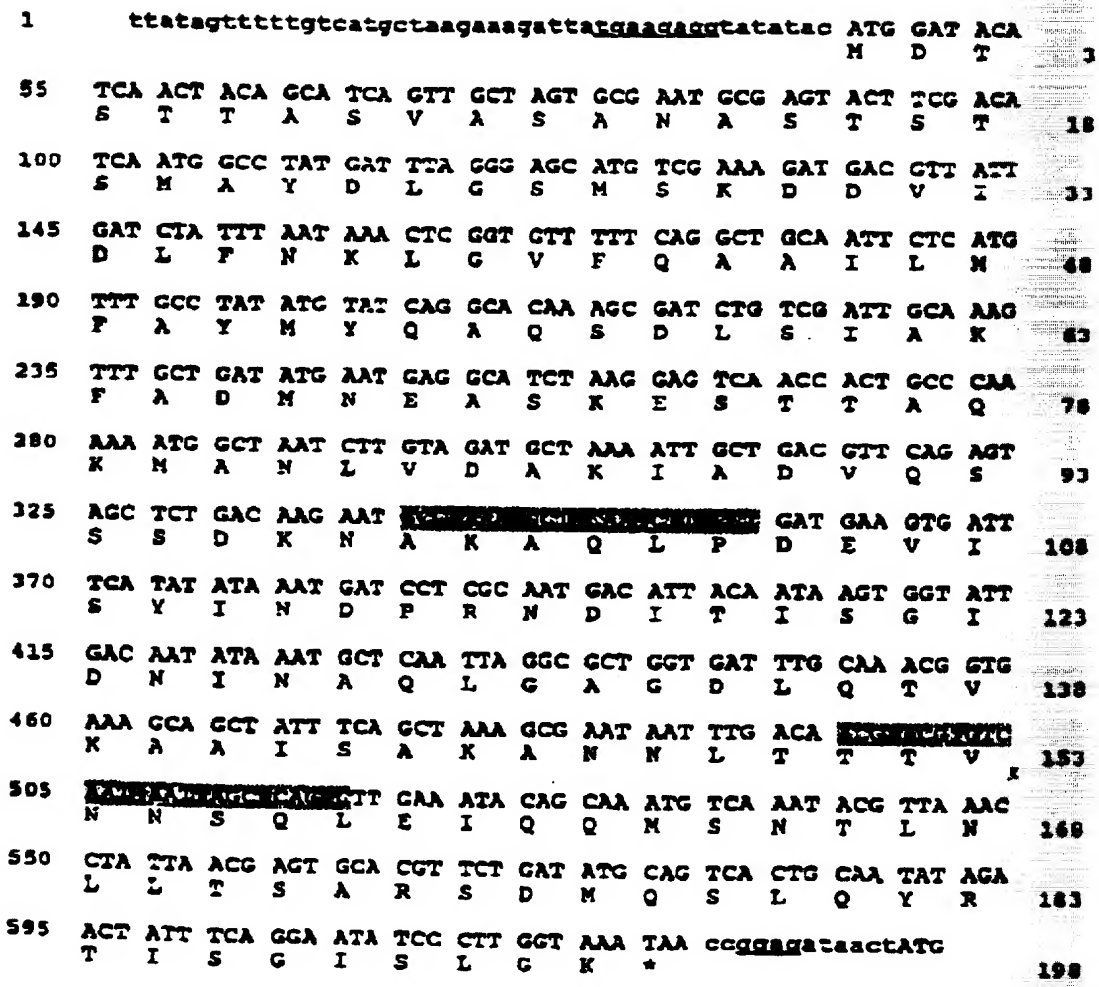
43. The kit of claim 42, wherein the label is selected from the group consisting of radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

44. A method of producing an EspA fusion protein comprising:

a) growing a host cell containing a polynucleotide encoding EspA operably linked to a polynucleotide encoding a polypeptide or peptide of interest under conditions which allow expression and secretion of the fusion protein; and

b) isolating the fusion protein.

FIGURE 1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S T T A S V A S A N A S T S T 18
100 TCA ATG GCC TAT GAT TTA GGG AGC ATG TCG AAA GAT GAC GTT ATT
S M A Y D L G S M S K D D V I 33
145 GAT CTA TTT AAT AAA CTC GGT GTT TTT CAG GCT GCA ATT CTC ATG
D L F N K L G V F Q A A I L M 48
190 TTT GCC TAT ATG TAT CAG GCA CAA AGC GAT CTG TCG ATT GCA AAG
F A Y M Y Q A Q S D L S I A K 63
235 TTT GCT GAT ATG AAT GAG GCA TCT AAG GAC TCA ACC ACT GCC CAA
F A D M N E A S K E S T T A Q 78
280 AAA ATG GCT AAT CTT GTA GAT GCT AAA ATT GCT GAC GTT CAG AGT
K M A N L V D A K I A D V Q S 93
325 AGC TCT GAC AAG AAT [redacted] GAT GAA GTG ATT
S S D K N A K A Q L P D E V I 108
370 TCA TAT ATA AAT GAT CCT CGC AAT GAC ATT ACA ATA AGT GGT ATT
S Y I N D P R N D I T I S G I 123
415 GAC AAT ATA AAT GCT CAA TTA GGC GCT GGT GAT TTG CAA ACG GTG
D N I N A Q L G A G D L Q T V 138
460 AAA GCA GCT ATT TCA GCT AAA GCG AAT AAT TTG ACA [redacted]
K A A I S A K A N N L T T T V K 153
505 [redacted] TT CAA ATA CAG CAA ATG TCA AAT ACG TTA AAC
N N S Q L E I Q Q M S N T L N 168
550 CTA TTA ACG AGT GCA CGT TCT GAT ATG CAG TCA CTG CAA TAT AGA
L L T S A R S D M Q S L Q Y R 183
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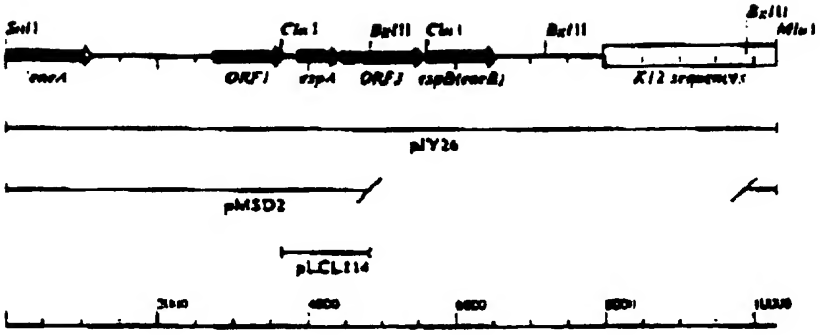
T I S G I S L G K * 198

FIGURE 2

A



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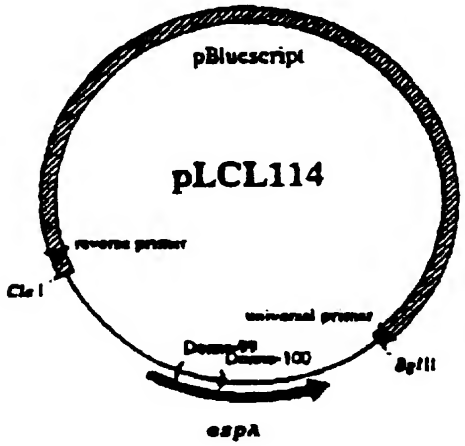

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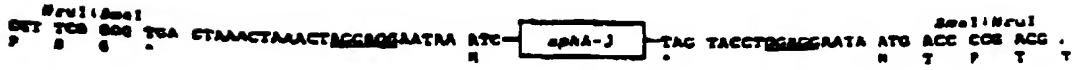
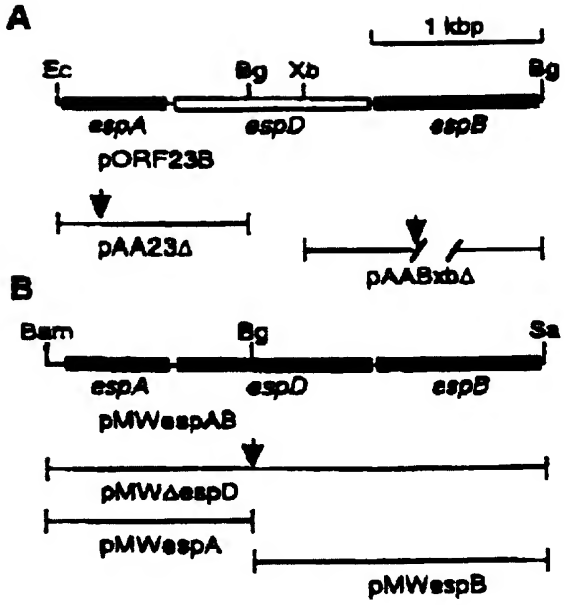


FIGURE 3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Bg
Xb
Bg
espA
espD
espB
pORF23B
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pAABxbΔ
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espD
espB
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pMWΔespD
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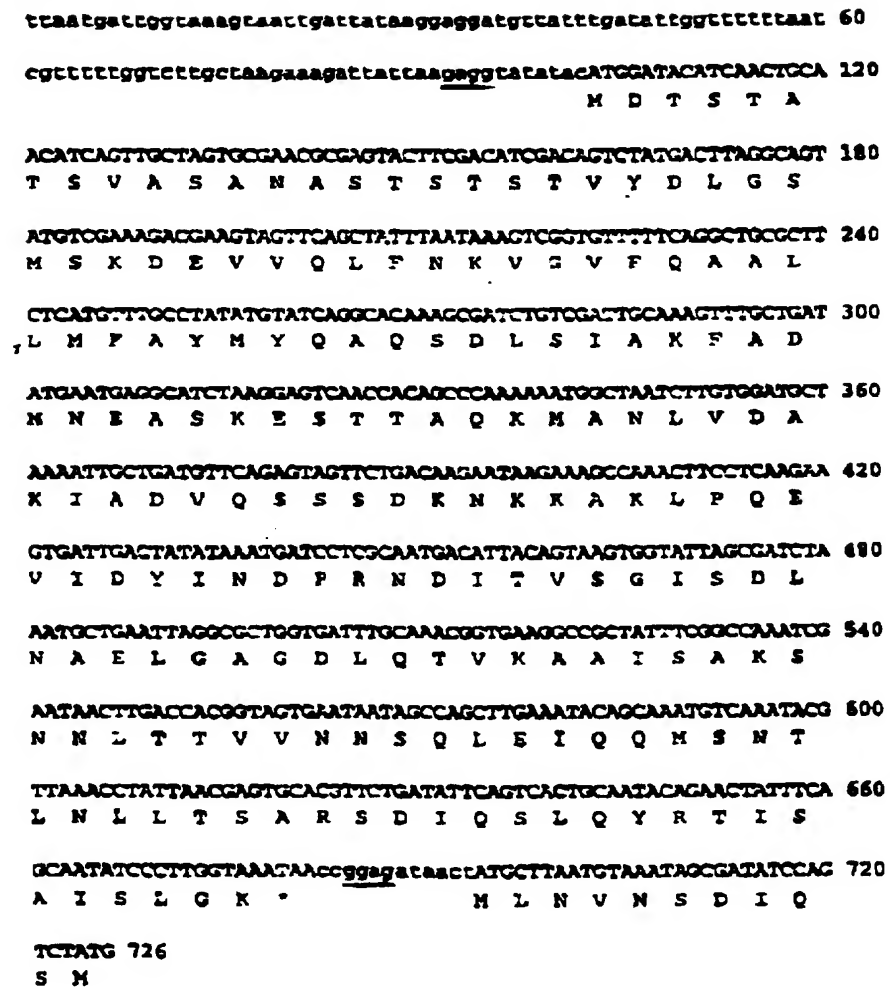
pMWespB

FIGURE 4A

A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T S V A S A N A S T S T S T V Y D L G S
ATGTCGAAAGACGAAGTAGTTCAGCTATTTAATAAAGTCGGTGTTTTTCAGGCTGCGCTT 240
M S K D E V V Q L F N K V G V F Q A A L
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L M F A Y M Y Q A Q S D L S I A K F A D
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M N E A S K E S T T A Q K M A N L V D A
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K I A D V Q S S S D K N K K A K L P Q E
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V I D Y I N D P R N D I T V S G I S D L
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L N L L T S A R S D I Q S L Q Y R T I S
GCAATATCCCTTGGTAAATAAccggagataactATGCTTAATGTAAATAGCGATATCCAG 720
A I S L G K * M L N V N S D I Q
TCTATG 726
S M

FIGURE 4B

B

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tcgagcttaattattaaagaggaaatttaattATGAATACTATTGATTATACTAATCAAGTA 60
                                  M  N  T  I  D  Y  T  N  Q  V

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M  T  V  N  S  V  S  E  N  T  T  G  S  N  A  I  T  A  S  A

ATTAATTCATCTTTGCTTACCGATGGTAAGGTGGATGTTTCTAAACTGATGCTGGAAATT 180
I  N  S  S  L  L  T  D  G  K  V  D  V  S  K  L  M  L  E  I

CAAAAACTCCTGGGCAAGATGGTGCGTATATTGCAGGATTACCAACAGCAACAGTTGTCG 240
Q  K  L  L  G  K  M  V  R  I  L  Q  D  Y  Q  Q  Q  Q  L  S

CAGAGCTATCAGATCCAACTGGCCGTTTTTGAGAGCCAGAATAAAGCCATTGATGAAAAA 300
Q  S  Y  Q  I  Q  L  A  V  F  E  S  Q  N  K  A  I  D  E  K

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K  A  A  A  T  A  A  L  V  G  G  A  I  S  S  V  L  G  I  L

GGCTCTTTGCAGCAATTAACAGTGCTACGAAAGGCGCGAGTGATATTGCTCAAAAACC 420
G  S  F  A  A  I  N  S  A  T  K  G  A  S  D  I  A  Q  K  T

GCCTCTACATCTTCTAAGGCTATTGATGCGGCTTCTGATACTGCGACTAAAACGTTGACT 480
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GTTACCAGTCTTGCCGAAGGCACGAAGACATTGCCAACAACGGTATCTGAATCAGTCAAA 780
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R  L  A  G  *
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FIGURE 4C